Overexpression of cyclooxygenase-2 (COX-2) in the mouse urinary bladder induces the expression of immune and cell proliferation related genes

Xingya Wang[1], Jennifer K. Colby[2], Susan M. Fischer[2], Russell D. Klein[1]

[1]Dept. of Human Nutrition and the Ohio State University Comprehensive Cancer Center, Cancer Chemoprevention Program, The Ohio State University, OH

[2]Dept. of Carcinogenesis, University of Texas, MD Anderson Cancer Center, Smithville, TX,

ABSTRACT

Bladder cancer is a common malignancy. Numerous studies have demonstrated that increased expression of cyclooxygenase enzyme 2 (COX-2), which catalyzes the synthesis of prostaglandins (PGs), is associated with the development and progression of certain type of cancers, including bladder cancer. However, the exact role of COX-2/PGs play in bladder carcinogenesis remains unknown. We have recently developed a transgenic mouse model which overexpresses COX-2 under the control of a bovine keratin 5 (BK5) promoter. The BK5.COX2 mice develop high incidence of bladder transitional cell hyperplasia (TCH) and a relative lower incidence of bladder papilloma. We then conducted a micorarray gene analysis to determine the effects of COX-2 overexpression on the regulation of gene expression profile in bladder carcinogenesis. Analysis of gene array data by t-test ($p<0.05$) revealed 70 of the upregulated genes and 60 of the downregulated genes had expression changed by 2-fold or more in transgenic compared to wild-type bladders. Gene set analysis using Expression Analysis Systematic Explorer software revealed that genes associated with Immune/Stress Response and Cell Cycle/Proliferation biologic processes were significantly overrepresented in the top 100 upregulated genes. Relevant downregulated genes included three TGF beta related genes (Tgfb2, Tgfb3, Tgfbi), and the anti-angiogenic gene thrombospondin 2 (Thbs2). We further demonstrated that the growth factor, epiregulin, is the most highly induced gene among the genes validated by real-time PCR. The results of gene expression analysis indicate that the initial response of the mouse bladder to elevated COX-2 expression includes mounting an inflammatory response and inducing cell proliferation. Future studies will focus on determining the role of the immune response in promoting or inhibiting the hyperplastic response of the bladder transitional epithelium. The role of epiregulin and the downstream signaling molecules in bladder carcinogenesis will also be determined. This study was supported by grants CA091865 and CA100140 from the National Cancer Institute.

METHODS

Materials. Polyclonal murine COX-1, COX-2 antibodies and PGE_2 EIA kit were from Cayman Chemical (Ann Arbor, MI). anti-rabbit HRP-linked IgG and HRP-conjugated anti-biotin antibody were from Cell signaling (Beverly, MA). Polyclonal beta-actin antibody was obtained from Abcam (Cambridge, MA). TRIzol reagent was purchased from Invitrogen (Carisbad, CA). RNeasy Mini-RNA extraction kit was purchased from Qiagen (Valencia, CA). The iScript cDNA synthesis kit was purchased from Bio-Rad (Hercules, CA). All primers (COX-1, COX-2, epiregulin, IGF-1, IGFBP6, TGFBi, TGFB2 TGFB3 and GAPDH) were purchased as Taqman® Gene Expression Assay kits from Applied Biosystems (Foster City, CA) as was the Taqman® Universal PCR Mastermix..

Animal Maintenance. Wild-type FVB mice and BK5.COX2 transgenic mice (FVB background) were kept in the University Laboratory Animal Resources (ULAR) facility of the Ohio State University (OSU) under an artificial day/night rhythm and fed chow diet and water ad libitum. Wild-type Female FVB mice were crossed with male BK5.COX2 mice to get the litters. Only female wild-type and transgenic mice were used for these studies.

Real-time PCR. Total RNA from age matched wild-type and BK5.COX2 mice was isolated from tissue using TRIzol reagent and further purified using RNeasy columns. RNA from three pooled wild-type mouse bladders was used as a calibration sample. Total RNA were subsequently analyzed with the Agilent Bioanalyzer 2100 system at the OSU CCC real-time PCR shared facility. Total RNA (1µg) was reverse transcribed with iScript cDNA synthesis kit . Real-time PCR was performed on a 1:10 dilution of the cDNA using Taqman gene expression primers and ABI mastermix according to the manufacturer's instructions on an iCycler IQ Real-Time PCR detection system (Bio-Rad). GAPDH was used as the reference gene for all samples.

Affymetrix microarray and EASE Analysis. Bladder tissues were excised from 10 wk old wild-type and BK5. COX-2 transgenic mice (5 mice/group). Total RNA were extracted by TRIzol reagent and then purified by RNeasy kit. The Affymetrix microchip analysis was performed by the Microarray Core Facility at the Columbus Children's Hospital Medical Center. Ten Mouse 430 GeneChips (Affymetrix Inc., Santa Clara, CA) were used; one chip for each sample. Affymetrix MicroArray Suite was used to scan and quantitate the genechips. Intensity data were collected from each chip and hybridization data were sequentially subjected to normalization, transformation, filtering, and functional classification. Differentially expressed genes in BK5.COX2 mice versus wild-type mice were identified by log2-differences and by t-test. The results were further analyzed using Expression Analysis Systematic Explorer (EASE; NIH) with the Benjamini false discovery rate correction for multiple testing.

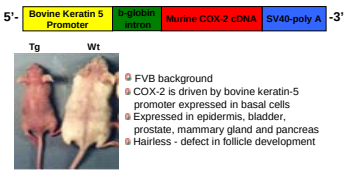
Tissue Preparation and Histological Analysis. Urinary bladders were excised and fixed in 10% Formalin. The fixed bladders were then bisected longitudinally through the urethral opening and positioned with the cut faces oriented in the same direction for paraffin embedding. Four-micron sections were stained with hematoxylin and eosin (H&E) by standard protocols. Immunohistochemical staining was carried out using modified ABC techniques. Primary antibody dilution for COX-2 was 1:2000 overnight at 4°C. Species appropriate biotinylated secondary antibodies were used at 1:200 for two hours at RT followed by ABC-HRP or SA-HRP treatment for 30 minutes at RT. Slides were developed with DAB for approximately five minutes. Mouse Ki-67 staining was performed by the histopathology core at OSU.

Immunoblot Analysis. Immunoblot analysis of COX-2, COX-1 and beta-actin were carried out according to the antibody manufacturer's instructions. Cell lysates from mouse ova deferens was used as positive control.

PGE_2 Measurement. PGE_2 concentrations in transgenic and wild-type mice were determined by PGE_2 EIA kit according to manufacturer's instruction from Cayman Chem.

Statistical Analysis. All data were analyzed by SPSS software. Values are means ± SD. Statistical significance ($p<0.05$) was determined by t-test.

BK5.COX2 MODEL



- FVB background
- COX-2 is driven by bovine keratin-5 promoter expressed in basal cells
- Expressed in epidermis, bladder, prostate, mammary gland and pancreas
- Hairless - defect in follicle development

OBJECTIVES

- To determine the effect of COX-2 overexpression on the gene expression profile in bladder epithelium.
- To explore potential mechanisms by which COX-2 can influence urinary bladder carcinogenesis.

INTRODUCTION

Bladder cancer represents an important heath concern. The identification of new molecular targets for bladder cancer prevention and therapy is a potential means to reduce the development and progression of this disease. Numerous studies have demonstrated that COX-2 is overexpressed in TCC of human urinary bladder [1-4]. The degree of COX-2 expression is significantly correlated with the tumor grade and depth of invasion (T stage) of TCC [5-7]. Increased COX-2 expression has also been reported in rat and canine models of bladder cancer [8, 9]. A case-control study involving 1514 incident bladder cancer cases and an equal number of controls indicated that NSAID (COX inhibitors) users have an almost 20% decreased risk for urinary bladder cancer [10]. A number of studies have demonstrated that NSAIDs are able to inhibit the growth of bladder cancer cells *in vitro* [11-14]. In addition, COX inhibitors also induced remission of chemically induced bladder tumors in rodents [15-16], naturally occurring invasive TCC in dogs [17-18], and bladder carcinoma in an orthotopic mouse model [19]. We have recently demonstrated that forced expression of COX-2, under the control of a keratin 5 (K5) promoter, is sufficient to cause urinary bladder transitional cell hyperplasia (TCH), which progresses to TCC in a small percentage of transgenic mice [20]. The level of prostaglandin E_2 (PGE_2), the major product of COX-2, was significantly increased in the bladder of BK5.COX2 mice. PGE_2 has also been reported to be elevated in patients with bladder cancer [4, 21].

The above studies suggest that there is a strong association between COX-2, prostaglandin synthesis and the development and progression of urinary bladder cancer. However, the exact role and mechanisms of COX-2 overexpression during bladder carcinogenesis have not been well defined. To determine the molecular events elicited by COX-2 overexpression during the early stage of bladder carcinogenesis (before the onset of substantial TCH), we conducted DNA microarray expression analysis on urinary bladders from 10 wk old wild-type and BK5.COX2 transgenic mice. Our study is innovative in that we were able to study gene expression changes that were due solely to the overexpression of COX-2 in the bladder tissue of a novel transgenic mouse model. This model may serve as a potentially useful model to study the etiology and prevention of human urinary bladder cancer.

RESULTS

COX-2 and COX-1 expression in the urinary bladder of BK5.COX2 transgenic mice

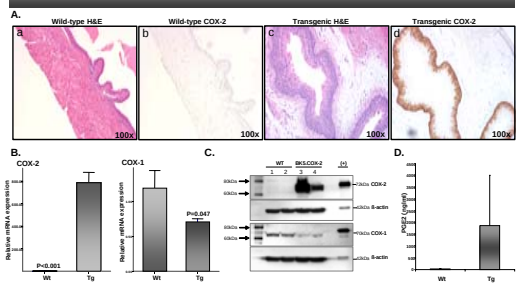


Figure 1. Confirmation of COX-2 transgene expression and PGE_2 production in BK5.COX2 transgenic mice. A. H&E staining and IHC staining with anti-COX-2. No COX-2 expression is observed in wild-type epithelial cells, whereas intense staining of COX-2 is present in the epithelial cells of transgenic bladders. **B.** qRT-PCR analysis. GAPDH was used as the reference gene. Results are represented as average expression relative to calibration sample (Error bars indicate the standard deviation, n=5, p<0.05). **C.** Immunoblotting analysis. 1-2: Wild-type; 3-4: Transgenic; (+): positive control. **D.** Elevated PGE_2 production in transgenic bladders compared to wild-type mice (Error bars indicate the standard deviation, n=4).

Transitional cell hyperplasia in BK5.COX2 transgenic mouse bladder tissue

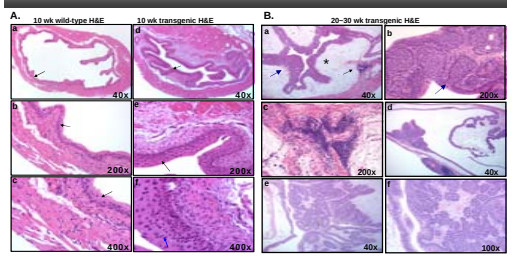


Figure 2. Histopathology of BK5.COX2 transgenic mouse bladder. **A**. Bladders from 10 wk old wild-type (a-c) and BK5.COX2 (d-f) mice. Note the thin epithelium (black arrow) in wild-type bladders in panel a, b and c. Note the simple TCH denoted by diffuse papillary extensions into the bladder lumen (d to f)) and thickened hyperplastic epithelium (black arrow in panel d and e), and increased hyperchromatic nuclei (f) and apoptotic cells (blue arrow in f). **B**. Bladders from older (20-30 wks) BK5.COX2 mice. Note the thickened lamina propria (* in a) and increased TCH, very often, the nodular hyperplasia (blue arrow in a) that characterized by the downward proliferation into the lamina propria and nests of epithelial cells (a-b). Note the nests of darkly strained inflammatory cells in panel **a** (black arrow) and panel c at higher magnification. Multifocal transitional cell papilloma and possible carcinoma in situ (d). Transitional cell papilloma (e-f), given more time, may progress to carcinoma.

Ki67 staining is increased in BK5.COX2

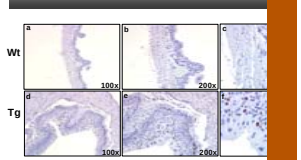


Figure 6. IHC staining of the proliferation marker Ki67 in 10 w type and BK5.COX2 transgenic urinary bladders. Note that th epithelial cells positive for Ki67 is significantly increased in bl transgenic mice (d-f) . Whereas, very few cells were stained Ki67 in the wild-type bladder epithelial cells (a-c, arrow in par

Regulation of gene expression profile by COX-2 overexpression in the BK5.COX2 mouse bladder at an early stage of carcinogenesis

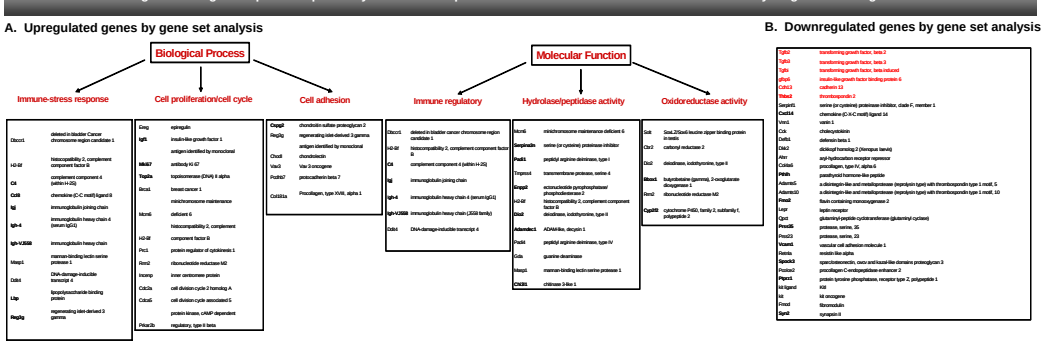


Figure 3. Affimatrix Microarray analysis of gene regulation in 10 wk old BK5.COX2 mice (n=5) compared to the wild-types (n=5). A. Upregulated genes; **B.** Downregulated genes. Analysis of gene array data by t-test (p<0.05) revealed that 2270 genes were upregulated and 1740 genes were downregulated in transgenic compared to wild-type mice. Seventy upregulated genes and sixty downregulated genes had expression changed by 2-fold or more (bold text) in transgenic compared to wild-type bladders. Gene set analysis of the array data using Expression Analysis Systematic Explorer (EASE; NIH) software determined that genes associated with Immune/Stress Response, Cell Cycle/Proliferation process, and Extracellular matrix remodeling were upregulated (A) (EASE<0.05; Benjamini<1.0); relevant downregulated genes include TGF-beta related genes (Tgfb2, Tgfb3, Tgfbi), and the anti-angiogenic gene thrombospondin 2 (Thbs2) (Panel B, red text).

Gene validation by qRT-PCR in 10 wk old mouse bladder tissues

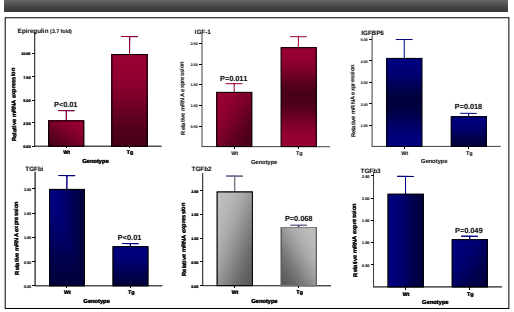


Figure 4. qRT-PCR validation of six genes of interest that were significantly regulated in 10 wk old BK5.COX2 transgenic mice by Gene Array analysis. Note that epiregulin is the most significantly upregulated gene (increased in BK5.COX2 by 3.7 fold, p<0.01). GAPDH was used as the reference gene. Results are represented as average expression relative to a calibration sample (Error bars indicate the standard deviation, n=5). As denoted by color, red: significantly upregulated genes, blue: significantly downregulated genes, grey: no significance (p<0.05).

Gene validation by qRT-PCR in 30 wk old mouse bladder tissues

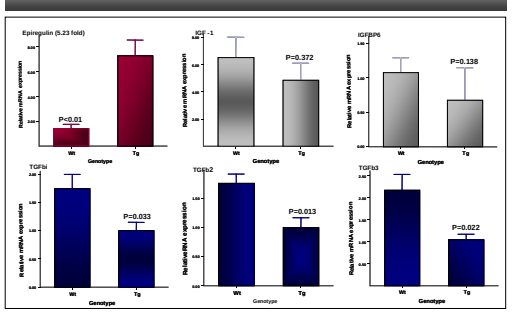


Figure 5. Expression of the six validated genes in 30 wk old BK5.COX2 transgenic mice and age matched wild-type mice by qRT-PCR analysis. Note that epiregulin is again the most significantly upregulated gene in 30 wk old animals (increased in BK5.COX2 by 5.3 fold, p<0.01). GAPDH was used as the reference gene. Results are represented as average expression relative to a calibration sample (Error bars indicate the standard deviation, n=5). As denoted by color, red: significantly upregulated genes, blue: significantly downregulated genes, grey: no significant change **(p<0.05)**.

CONCLUSIONS

- COX-2 overexpression induces the re of genes involved in immune/stress, antiangiogenic, proliferation/cell cycle extracellular matrix remodeling events initiating responses.
- The growth factor, epiregulin, is the m significantly upregulated gene as valid qRT-PCR.
- The expression of epiregulin and the T beta family were consistently deregula the progression of urinary mouse blad carcinogenesis.
- BK5. COX2 may serve as a mouse m human bladder carcinogenesis.
- Biomarkers of COX-2 overexpression useful in future translational chemopre studies focusing on bladder cancer.

FUTURE DIRECTION

- Determine the presence and level o prostaglandins and their respective re
- Determine the role of epiregulin downstream signaling molecules in carcinogenesis.
- Examine the interaction between CO TGF-beta signaling pathways in carcinogenesis.
- Explore the role of the immune resp regulating the hyperplastic response bladder transitional epithelium.

Acknowledgements: We would like to thank for Rob Rengel for his help with Sargeant (Veterinary pathologist) for histopathologic evaluation.

References:

[1]. Sweeney, C.J., et al., Cyclo-oxygenase-2 expression in primary cancers of the lung and normal adjacent tissue. Cancer Detect Prev, 2002. 26(3): p. 238-44.

[2]. Shirahama, T., Cyclooxygenase-2 expression is up-regulated in transitional cell carcino lesions in the human urinary bladder. Clin Cancer Res, 2000. 6(6): p. 2424-30.

[3]. Mohammed, S.I., et al., Expression of cyclooxygenase-2 (COX-2) in human invasive tra (TCC) of the urinary bladder. Cancer Res, 1999. 59(22): p. 5647-50.

[4]. Hara, S., et al., Expression of cyclooxygenase-2 in human bladder and renal cell carcino 2002. 507: p. 123-6.

[5]. Gurocak, S., et al., Relation between cyclooxygenase-2 expression and clinicopathologic patient prognosis in TCC of the bladder. Urol Int, 2006. 76(1): p. 51-6.

[6]. Wadhwa, P., et al., Cyclooxygenase-2 expression increases with the stage and grade in carcinoma of the urinary bladder. Int Urol Nephrol, 2005. 37(1): p. 47-53.

[7]. Wulfing, C., et al., Cyclooxygenase-2 expression in bladder cancer: correlation with poor chemotherapy. Eur Urol, 2004. 45(1): p. 46-52.

[8]. Khan, K.N., et al., Expression of cyclooxygenase-2 in transitional cell carcinoma of the u Am J Vet Res, 2000. 61(5): p. 478-81.

[9]. Kitayama, W., et al., Increased expression of cyclooxygenase-2 protein in rat urinary bla N-butyl-N-(4-hydroxybutyl) nitrosamine. Carcinogenesis, 1999. 20(12): p. 2305-10.

[10]. Castellsa, J.E., et al., Non-steroidal anti-inflammatory drugs and bladder cancer preven 82(7): p. 1364-9.

[11]. Gee, J., et al., Selective cyclooxygenase-2 inhibitors inhibit growth and induce apoptos Oncol Rep, 2006. 15(2): p. 471-7.

[12]. Mohsin, H., et al., COX-2 inhibition demonstrates potent anti-proliferative effects on bl Surg Res, 2004. 119(2): p. 138-42.

[13]. Mizutani, Y., et al., Enhanced sensitivity of bladder cancer cells to cisplatin mediated cy in vitro and in vivo by the selective cyclooxygenase-2 inhibitor JTE-522. J Urol, 2004. 172(4

[14]. Mizutani, Y., et al., Synergistic cytotoxicity and apoptosis of JTE-522, a selective cyclo and 5-fluorouracil against bladder cancer. J Urol, 2002. 168(6): p. 2650-4.

[15]. Okajima, E., et al., Chemopreventive effects of nimesulide, a selective cyclooxygenase- Development of rat urinary bladder carcinomas initiated by N-butyl-N-(4-hydroxybutyl)nitrosa 1998. 58(14): p. 3028-31.

[16]. Grubbs, C.J., et al., Celecoxib inhibits N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced in male B6D2F1 mice and female Fischer-344 rats. Cancer Res, 2000. 60(20): p. 5599-602.

[17]. Knapp, D.W., et al., Cisplatin versus cisplatin combined with piroxicam in a canine mod urinary bladder cancer. Cancer Chemother Pharmacol, 2000. 46(3): p. 221-6.

[18]. Mohammed, S.I., et al., Effects of the cyclooxygenase inhibitor, piroxicam, on tumor re angiogenesis in a canine model of human invasive urinary bladder cancer. Cancer Res, 200

[19]. Smakman, N., et al., NS-398, a selective cyclooxygenase-2 inhibitor, reduces experime outgrowth by inhibiting tumor cell proliferation. Urology, 2005. 66(2): p. 434-40.

[20]. Klein, R.D., et al., Transitional cell hyperplasia and carcinomas in urinary bladders of t keratin 5 promoter-driven cyclooxygenase-2 overexpression. Cancer Res, 2005. 65(5): p. 18

[21]. Badawi, A.F., et al., Influence of cigarette smoking on prostaglandin synthesis and CO human urinary bladder cancer. Cancer Invest, 2002. 20(5-6): p. 651-6.